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Female genital tract-delivery of short-interfering RNA inhibits mucosal transmission of HIV-1 in humanized mice

Susan K. Eszterhas[1,2], Sherrill L. Macura[1], Kristen L. Flint[4], Jennifer L. Fields[2], Steven N. Fiering[2] and Alexandra L. Howell[1,2,3,*]

[1]Department of Veterans Affairs, Veterans Health Administration, Biomedical Laboratory Research and Development, White River Junction, VT 05009; [2]Departments of Microbiology & Immunology and [3]Medicine, Geisel School of Medicine at Dartmouth, Lebanon, NH 03756; [4]Dartmouth College, Hanover, NH 03755, USA.

ABSTRACT

Current therapies to block the sexual transmission of HIV-1 have utilized microbicidal or antiviral compounds to interfere with viral binding, internalization, or replication. These approaches, however, have met with only limited success. Thus, there is a critical demand to develop novel therapeutics to inhibit mucosal HIV-1 infection and transmission. We examined the efficacy of short interfering RNA (siRNA) molecules to silence expression of CD4 and CCR5 on cells in the reproductive tract of female humanized mice. We postulated that silencing expression of these receptors could inhibit HIV-1 binding and infection. Nanoparticles encapsulating siRNAs to silence CD4 and CCR5 expression, or an irrelevant siRNA, were instilled intravaginally in mice. Three days later the mice were challenged intravaginally with HIV-1. Results demonstrated significant reduction in HIV-1 DNA levels in the peripheral blood regardless of siRNA specificity. Elevated levels of both murine and human TNF-α transcripts were detected in the genital tracts of mice following siRNA delivery. These findings suggest that nucleic acid-based therapies may have the unintended, although highly advantageous, effect of triggering innate immune anti-viral mechanisms. Thus, the development of nucleic acid based-therapies such as the one using siRNA that exploits this potent additive anti-HIV-1 response while concurrently targeting specific HIV-1 recognition receptors, is highly desirable and could be used to supplement microbicidal or antiviral therapies.

KEYWORDS: RNA interference, Human immunodeficiency virus-type 1, microbicide, mucosal transmission, innate immunity, toll-like receptor

INTRODUCTION

The development of microbicidal compounds to inhibit the transmission of HIV-1 from mucosal tissues to the periphery has largely relied on the use of agents that either directly destroy viral particles, or interfere with one or more steps in the viral life-cycle [1]. However, despite the development and testing of several microbicidal agents in eleven clinical trials over the past twenty years, no single microbicide has consistently demonstrated effectiveness in study participants [2]. Microbicides composed of anti-retroviral compounds served as the next generation of agents tested in clinical trials, yet had mixed success in reducing HIV-1 transmission in study participants. The CAPRISA 004 trial of 1% tenofovir in gel form was found to be

*Corresponding author: Alexandra L. Howell, Ph.D.
Research Service, V. A. Medical Center,
215 North Main St., White River Jct., VT 05009, USA.
Alexandra.L.Howell@Dartmouth.edu

modestly effective in women who used the gel prior to sexual intercourse [3, 4], reducing transmission rates by 39% compared to placebo controls. Therefore, a critical demand remains to develop novel therapeutics to inhibit HIV-1 infection and transmission.

The use of animals to perform pre-clinical testing of microbicidal and other anti-HIV compounds has been widely accepted to be a suitable and valid approach to identify potentially effective agents that would then be selected for clinical testing [5]. Non-human primates including macaques have served as an animal model for studies of HIV vaccines [6, 7], anti-retroviral chemotherapy [8, 9], as well as microbicides [10-12]. More recently, improvements to the generation of human progenitor cell-engrafted murine models (humanized mice) have yielded a small animal model that mimics the course of HIV-1 infection in humans more closely than was observed with SIV infection in non-human primates [13, 14]. A recent publication by Garcia-Martinez demonstrated that the bone marrow-liver-thymus (BLT) model of humanized mice were valid models to test the efficacy of a 1% tenofovir microbicide applied rectally [15].

Small interfering RNAs (siRNAs) have been powerful research tools for the specific knock-down of proteins. The promise of reducing *in vivo* the level of cell surface receptors and other proteins critical in HIV infection has lead us and others to investigate the use of siRNA as a therapeutic to block mucosal transmission of HIV. Wheeler *et al.* prepared a hydroxyethylcellulose gel formulation containing a CD4-binding aptamer-siRNA complex to silence the expression of the cellular protein CCR5, and the HIV-1 proteins Gag and Vif, and delivered this complex intra-vaginally in humanized mice [16]. These authors found that vaginal transmission of HIV-1 was completely blocked for several days after delivery of the CD4 aptamer-multi-siRNA complex. In a related study, Akkina *et al.* showed that the vaginal application of a gel containing broadly neutralizing antibodies could protect humanized mice from HIV-1 transmission if the antibodies were applied one hour prior to viral challenge [17]. These publications demonstrate the feasibility and suitability of the use of both siRNA and the humanized murine model to test the efficacy of anti-HIV-1 therapies, and suggest that pre-clinical testing of potential agents can not only define efficacy, but can assist in determining the mechanism of action.

Our current studies evaluated the extent to which siRNA molecules that silence the expression of HIV-1 cell receptors CD4 and CCR5 on target cells in the female genital tract of humanized mice could protect against vaginal transmission of HIV-1. We used female humanized mice and encapsulated the siRNA constructs in nanoparticles. Herein we report that the delivery of either receptor-specific or irrelevant siRNA nanoparticles significantly inhibited HIV-1 DNA levels in the peripheral blood from mice infected intravaginally with cell-free HIV-1_{BaL}, an R5-tropic strain of HIV-1. These findings indicate that mechanisms in addition to receptor silencing are contributing to protection from vaginal transmission of HIV-1. Moreover, our findings show that the vaginal instillation of nanoparticles containing siRNA induces expression of a potent anti-viral compound, tumor necrosis factor-alpha (TNF-α) [18, 19]. Thus, nanoparticles encapsulating siRNA applied to the mucosal tissues of the genital tract is a viable approach to inhibit vaginal transmission of HIV-1, and may function to activate innate immune responses perhaps by toll like receptor (TLR) signaling. The use of nanoparticles to activate innate anti-viral immune responses could be developed into a potent nucleic acid-based therapy for a host of sexually transmitted pathogens including HIV-1. This approach may have advantages over existing topically applied microbicidal compounds with the potential to eliminate adherence issues that have reduced the efficacy of current therapies in clinical trials.

MATERIALS AND METHODS

Humanized mice

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, and were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at both the Geisel School of Medicine and the Department of Veterans Affairs (Protocol # 00401-12). All

procedures were carried out under isofluorane anesthesia, and all efforts were made to minimize animal suffering. Carbon dioxide asphyxiation was used to carry out euthanasia at the completion of the study. Six- to 8-week old NOD/SCID/Il2 receptor gamma chain knockout mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, stock #5557), hereafter referred to as NSG, were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred in the animal facility at the Geisel School of Medicine at Dartmouth. NSG mice lack murine B, T and natural killer cells. These mice were maintained in cages ventilated with high-efficiency particulate arrestance (HEPA)-filtered air and fed sterile food (Harlan Teklad #2919, South Easton, MA) and water supplemented with the antibiotic sulfamethoxazole/trimethoprim (Septra®, 150 mg/30 mg per 100 ml water, HiTech Pharmacal, Amityville, NY). For this study, humanized female mice between the ages of 8 to 10 weeks were obtained from the Transgenic Animal Facility at the Geisel School of Medicine, Lebanon, NH. In brief, mice were engrafted with human CD34+ hematopoietic progenitor cells according to the method of Dr. Andrew Tager [20]. All donor cells were screened for the absence of the delta-32 mutation in the CCR5 gene by polymerase chain reaction (PCR) agarose gel electrophoresis. The percentage of human CD45 cells present in the peripheral blood of each mouse was determined at 12-weeks post-engraftment by antibody incubation of peripheral blood followed by flow cytometric analysis. In brief, peripheral blood was obtained from each mouse by retro-orbital bleeding and anti-coagulated with heparin. Blood was incubated with a fluorescein-conjugated anti-human CD45 (Biolegend, San Diego, CA) for 20 minutes. Red blood cells were lysed and the cell suspension was washed with phosphate buffered saline containing 1% bovine serum albumin and analyzed by flow cytometry. Controls included cells incubated with murine antibodies of irrelevant specificity (isotype controls). The leukocyte fraction was gated and the percentage and mean fluorescence intensity of the positive cells was determined.

Reagents

siRNA specific for human CD4 (s225098), CCR5 (s3211), or an irrelevant sequence (4390843), were obtained from Applied Biosystems/Ambion (Carlsbad, CA). The encapsulating/transfection reagent *in vivo*-jetPEI was obtained from Genesee Scientific (San Diego, CA). *In vivo*-jetPEI carries a net positive charge, and when combined with the negatively charged siRNA molecules under vigorous vortexing forms nanoparticles that encapsulate the siRNA as demonstrated by Cubillos-Ruiz *et al.* [21]. In the absence of a negatively charged nucleic acid, the *in vivo*-jetPEI does not form a nanoparticle, and remains as a linear polyethylene imine fragment.

Encapsulation of siRNA into nanoparticles

Nanoparticles encapsulating siRNA molecules were prepared by vortexing a solution containing 33 µM siRNA in 5% glucose and 17.5% *in vivo*-jetPEI followed by 20 min incubation at room temperature. The final N/P ratio, the molar ratio of cationic lipid nitrogen (N) to siRNA phosphate (P), was 8. This suspension was designed to contain 1 nmole siRNA plus 5.3 µl *in vivo*-jetPEI per 30 µl.

siRNA nanoparticle instillation into humanized mice

Thirty µl of the suspension containing nanoparticles with equimolar concentrations of CD4 and CCR5 specific siRNA, or irrelevant siRNA, or an equivalent volume of the glucose/water diluent, was instilled into the vaginal vault directly to the cervical os while directing the solution first into one uterine horn and then into the other uterine horn. Mice were anesthetized with inhalation of isofluorane immediately prior to and during the instillation, and were kept anesthetized and in a head down position for 5 minutes afterwards to prevent the solution from exiting the vaginal canal. Mice were monitored while recovering from anesthesia before being returned to their cages.

HIV-1 infection of humanized mice

Three days after the instillation of siRNA nanoparticles into the vaginal canal, the mice were infected with cell-free HIV-1_{BaL}, an R-tropic strain of HIV-1. Viral stocks of HIV-1_{BaL} were stored at -80 °C, and were thawed at room temperature and sonicated for two minutes immediately prior to use. Each mouse was anesthetized by isofluorane inhalation and then 40 µl of virus (equivalent to a

dose of 50 $TCID_{50}$) was instilled via a sterile plastic pipette tip directly into the vaginal tract as was done with the siRNA nanoparticles. Mice were kept anesthetized and in a head down position for 5 minutes afterwards to prevent the solution from exiting the vaginal canal. Mice were monitored while recovering from anesthesia before being returned to their cages.

Quantification of HIV-1 infection in humanized mice

On weeks 2, 3, 4, 5 and 6 following HIV-1 instillation, peripheral blood was obtained from each mouse for analysis of HIV-1 DNA by PCR. The day before the blood draw, and for each of the next two days, sterile water supplied to each cage (200 ml) was supplemented with 4.7 ml of children's liquid ibuprofen (Motrin®) to mitigate any potential pain resulting from the blood collection procedure. On the day of the blood collection, mice were anesthetized by isofluorane inhalation, and a 1-2 mm section of tail was removed by sterile scalpel. Under anesthesia, a single drop of blood (approximately 35 µl) was collected onto sterile Whatman filter paper and allowed to dry in a laminar flow hood overnight. Thereafter the dried blood spots were stored at -80 °C.

Genomic DNA was isolated from the dried blood spots using Qiagen's QiaAmp DNA Blood mini kit (catalog#51104, Qiagen) according to the method of Mehta *et al.* [22]. In brief, the dried blood spots were cut out from the Whatman filter paper, sectioned into 2-3 mm slivers and placed into a microfuge tube. Two hundred microliters of a tissue lysis buffer (ATL) was added, and the preparation was incubated at 85 °C for 10 minutes with gentle shaking and occasional vortexing. This was followed by the addition of 20 µl of Proteinase K, and further incubation for 1 hr at 56 °C. Two hundred µl of a second lysis buffer (AL) was then added for 10 minutes at 70 °C. Thereafter, the manufacturer's protocol was followed, eluting the genomic DNA from the column with 150 µl Tris-EDTA (buffer AE). DNA concentration was determined by UV absorption on a Nanodrop™ spectrophotometer.

HIV-1 DNA levels in the peripheral blood were quantified by real-time PCR. Each PCR replicate contained 60 ng of genomic DNA, IQ SYBR Green Supermix (BioRad, Hercules, CA), and 0.4 µM primers to the HIV-1 LTR: forward primer: 5'-GGAACCCACTGCTTAAGCCTCAA-3' and reverse primer: 5'-TGTTCGGGCGCCACTGCTAGAGA-3' (derived from [22, 23]). The reactions were normalized to total DNA with primers that detected both human and mouse β-actin DNA, forward: 5'-GAGGCCCAGAGCAAGAGAG-3' and reverse: 5'-TGTAGAAGGTGTGGTGCCAGAT-3'. The total volume of each PCR reaction was 25 µl, and all the real time PCR reactions were run in triplicate.

Quantification of cell receptor, cytokine and anti-viral gene expression

To study the regulation of gene expression resulting from siRNA delivery, nanoparticles containing siRNA were instilled intra-vaginally in the genital tract of female humanized mice as described above. Groups of mice were euthanized on day 1, 3 or 5 post-instillation, and the entire genital tract was removed and placed in RNA later (Qiagen, Valencia, CA). Tissues were minced into small pieces using sterile scalpels, and RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). To remove contaminating genomic DNA, an on-column DNAse digestion was performed according to the manufacturer's specifications (Qiagen). Two µg of total RNA from each time point was reverse transcribed to cDNA using random hexamer primers in a 50 µl reaction volume as described in [24]. Primers to human CCR5 (forward: GGC CAG AAG AGC TGA GAC ATC C, reverse: CGG GCT GCG ATT TGC TTC ACA T), human CD4 (forward: GGG GAT ACA GTG GAA CTG ACC, reverse: TCC CAA AGG CTT CTT CTT GAG), and human CD45 (forward: CAC GGC TGA CTT CCA GAT ATG A, reverse: GGT GCT TGC GGG TGA GAA T) were used to quantify transcript levels in siRNA-exposed tissues. Primers to human myxovirus resistance protein 1 (MxA, forward: GCT ACA CAC CGT GAC GGA TAT GG, reverse: CGA GCT GGA CTG GAA AGC CC), 2,5-oligoadenylate synthetase (OAS, forward: CAT CCG CCT AGT CAA GCA CTG, reverse: CCA CCA CCC AAG TTT CCT GTA G), Protein kinase RNA-activated (PKR, forward: AAG CAA AAC AAT TGG CCG CT, reverse: GTT GCT TTG GGA CTC AGA CG),

human and murine tumor necrosis factor-alpha (TNF-α, human forward: CCC CAG GGA CCT CTC TCT AAT, human reverse: GTC TGG TAG GAG ACG GCG AT, murine forward: TCT ACT GAA CTT CGG GGT GAT CG, murine reverse: ACG TGG GCT ACA GGC TTG TCA), and murine and human interferon-alpha (IFN-α, human forward: CCC AAAG GTT CAG AGT CAC CC, human reverse: ACT CCT CCT GGG GAA ATC CA, murine forward: GCT AGG CHY TRT GCT TTC CT, murine reverse: CAC AGT GGC TGT GTT TCT TC) were used to quantify transcript levels from siRNA-exposed tissues. Transcript levels were normalized to the amount of cDNA with primers that detect both human and murine GAPDH transcripts (forward: TTC ACC ACC ATG GAG AAG GGC, reverse: GGC ATG GAC TGT GGT CAT GA).

Statistical analysis

Analysis of datasets comparing two groups was performed by student's t-test, and by comparing multiple groups by ANOVA, and were considered statistically significant at $p \leq 0.05$.

RESULTS

Human hematopoietic cell engraftment in NOD SCID gamma (NSG) mice

At 12 weeks following CD34+ cell infusion, peripheral blood was obtained from each animal and analyzed for the percentage of human CD45+ (hCD45+) cells in the white blood cell fraction. We selected mice that achieved at least 10% hCD45 by 12 weeks post-engraftment for these experiments (Table 1). As depicted in Table 1, mice were included in the treatment groups such that the average and standard error of the mean of CD45 expression for each group was similar to the other groups in that experiment. Table 1 shows the mouse ID#, type of siRNA treatment, and the percentage of huCD45+ cells at 12 weeks. We used 8 or 9 mice per experimental group.

Timeline of experimental design

The overall schema of the experimental protocol is depicted in Figure 1. In brief, the percentage of human CD45+ cells in the peripheral blood was measured at 12 weeks post-implantation as described above. Mice selected for this study then received an intravaginal instillation of siRNA nanoparticles suspended in a glucose diluent, and were either infected three days later with HIV-1 or euthanized at various times post-siRNA treatment for analysis of expression levels of leukocyte receptors, cytokines and anti-viral genes. The data from each group of mice based on treatment is shown in Figures 2 to 5.

HIV-1 transmission is inhibited following siRNA delivery via nanoparticles

Female humanized mice received an intra-vaginal instillation of nanoparticles encapsulating siRNA specific for CD4 and CCR5, or an irrelevant siRNA, and were then challenged intravaginally with the R5 tropic strain, HIV-$1_{Ba\text{-}L}$. Control mice received an intravaginal instillation of a glucose solution that was the dilutent for the *in vivo*-jetPEI. Peripheral blood samples were obtained from each mouse starting at week 2, and applied to a piece of Whatman filter paper and allowed to dry. DNA was extracted from the filter paper and levels of HIV-1 DNA as a surrogate marker for circulating HIV-infected cells were measured by PCR [22]. This method of HIV-1 detection provides a more specific evaluation of viral transmission to the periphery than analysis of blood plasma levels of p24 antigen [25]. Peak levels of HIV-1 DNA were plotted for each animal in each experimental or control group. HIV-1 DNA levels were expressed relative to the earliest time of HIV-1 DNA detection (day 12), which was arbitrarily assigned a value of "1". As shown in Figure 2, the mean peak HIV-1 DNA levels in the control group ranged from 12 to 96, with a mean ($\pm$SEM) of 47.1 ($\pm$5.9). In contrast, mice that received the CD4 and CCR5-specific siRNAs showed a much lower peak values with a mean of 8.7 $\pm$ 1.0, and mice that received the irrelevant siRNA also had low HIV-1 DNA peak levels, with a mean of 7.9 $\pm$ 1.0.

Expression of CD4, CCR5 and CD45 in the female genital tract of mice treated with siRNAs

The intra-vaginal instillation of CD4 and CCR5-specific siRNA was designed to inhibit expression of CD4 and CCR5 and thereby reduce mucosal HIV-1 infection and subsequent transmission of virus to the periphery by eliminating infectivity receptors on target cells. To assess the extent of

Table 1A. Mice used in HIV-1 infection analyses.

ID#[1]	siRNA treatment[2]	Percent huCD45[3]	ID#	siRNA treatment	Percent huCD45	ID#	siRNA treatment	Percent huCD45
842	glucose control	25.5	845	CCR5 & CD4	11.1	843	irrelevant	10.8
897	glucose control	18.6	846	CCR5 & CD4	36.2	892	irrelevant	12.2
1851	glucose control	10.6	848	CCR5 & CD4	10.9	894	irrelevant	34.4
1885	glucose control	24.4	1886	CCR5 & CD4	16.2	1888	irrelevant	16.5
3201	glucose control	28.9	1887	CCR5 & CD4	30.0	1889	irrelevant	39.8
3202	glucose control	33.5	1892	CCR5 & CD4	21.6	1890	irrelevant	17.2
3203	glucose control	36.4	3250	CCR5 & CD4	35.3	2645	irrelevant	35.0
3246	glucose control	32.2	3251	CCR5 & CD4	36.6	3252	irrelevant	32.8
			3253	CCR5 & CD4	30.1			
	Ave. %[4]	**26.3(±3.0)**		**Ave. %**	**25.3(±3.5)**		**Ave. %**	**24.8(±4.2)**

Table 1B. Uninfected mice for transcription analyses.

Day 1 ID#	siRNA treatment	Percent huCD45	Day 3 ID#	siRNA treatment	Percent huCD45	Day 5 ID#	siRNA treatment	Percent huCD45
6020	glucose control	56.9	6085	glucose control	35.2	4195	glucose control	20.7
6040	glucose control	40.4	6088	glucose control	35.3	4239	glucose control	50.9
6057	glucose control	36.2	6093	glucose control	26.2	4240	glucose control	38.9
	Ave. %	**44.5(±6.3)**		**Ave. %**	**32.2(±3.0)**		**Ave. %**	**36.8±(8.8)**
5695	CCR5 & CD4	56.0	6095	CCR5 & CD4	27.8	4203	CCR5 & CD4	34.8
6058	CCR5 & CD4	47.6	6097	CCR5 & CD4	38.6	4264	CCR5 & CD4	37.7
6197	CCR5 & CD4	32.1	6102	CCR5 & CD4	16.3	4618	CCR5 & CD4	44.5
6201	CCR5 & CD4	45.6						
	Ave. %	**45.3(±5.0)**		**Ave. %**	**27.6(±6.4)**		**Ave. %**	**39(±2.9)**
5685	irrelevant	55.0	6087	irrelevant	19.0	4245	irrelevant	38.9
6038	irrelevant	49.6	6103	irrelevant	27.7	4248	irrelevant	37.2
6041	irrelevant	41.6	6104	irrelevant	46.0	4265	irrelevant	50.7
6199	irrelevant	32.9						
	Ave. %	**44.8(±4.8)**		**Ave. %**	**30.9(±8.0)**		**Ave. %**	**42.3(±4.3)**

[1]ID#: the unique identification number given to humanized mice.
[2]siRNA treatment: siRNAs were instilled intravaginally as nanoparticles made with *in vivo*-jetPEI suspended in 5% glucose. The control mice received 5% glucose intravaginally.
[3]Percent huCD45: percentage of peripheral blood cells positive for human CD45 as determined by flow cytometry at 12 weeks post-CD34+ cell infusion.
[4]Ave.: mean percentage of huCD45 within the group (plus or minus the standard error of the mean).

human CD4 and CCR5 transcript level reduction by exposure to receptor-specific siRNA, we quantified levels of mRNA to CD4 and CCR5 in genital tract tissues on days 1, 3 and 5 after instillation. Levels of CCR5 or CD4 transcripts were arbitrarily set to "1" in the control mice for each day of analysis (Figure 3A and 3B). In mice that received either the CD4/CCR5 combination

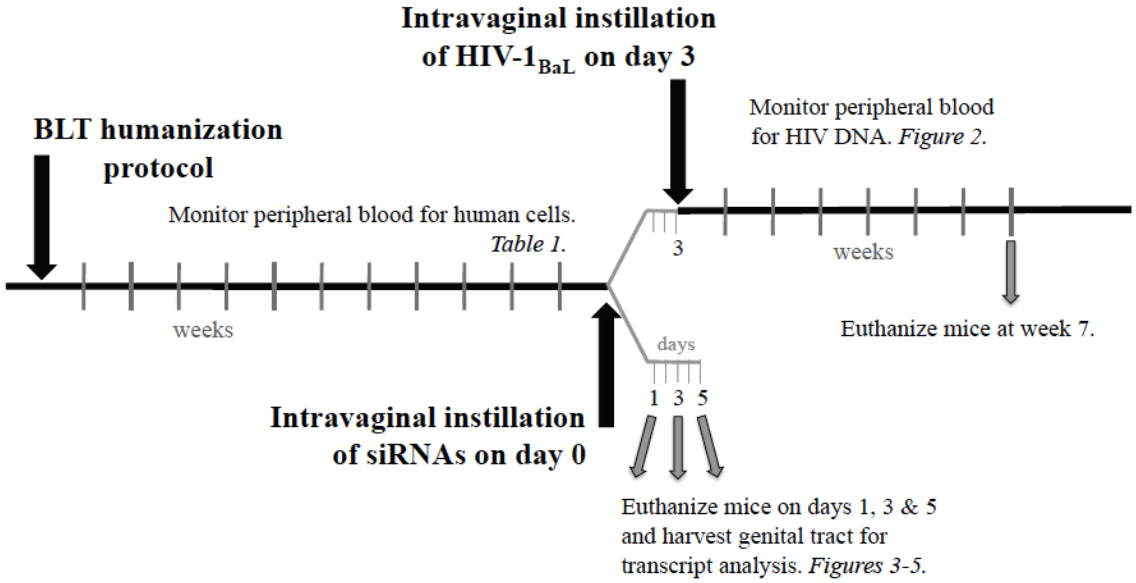

Figure 1. Timeline of experimental protocol. NSG immunodeficient mice were engrafted with human CD34+ hematopoietic progenitor cells. Mice were assessed for the percentage of human CD45+ cells in the peripheral blood on week 12 and then assigned to one of several experimental or control groups. At the time of siRNA instillation, all mice were between 5 and 6 months of age. Mice received either glucose (control), or siRNAs to CD4 and CCR5, or an irrelevant siRNA sequence. These mice were used to assess expression of inflammatory mediators and receptor expression on genital tract tissues. Some of the mice also were challenged with HIV-$1_{Ba\text{-}L}$ on day 3 post-siRNA instillation and were monitored for levels of HIV-1 DNA weekly, starting from week two for 7 weeks.

of siRNAs or the irrelevant siRNA, CCR5 transcript levels declined throughout the 5-day period (Figure 3A). However, the decrease in CCR5 reached statistical significance only on day 5 (Figure 3A). For CD4, the same pattern of decrease in transcript levels was observed, but due to inter-animal variability, this did not achieve statistical significance (Figure 3B).

To determine whether the decreases in CD4 and CCR5 transcript levels in siRNA-treated mice were due to a reduction in the number of leukocytes present in the genital tract after siRNA instillation, we measured human CD45 transcript levels in genital tract tissues from all mice. By 24 hr post-siRNA instillation, we observed an increase in CD45 transcripts in mice that received siRNA regardless of the specificity of the siRNA, although this was not statistically significant compared to the control treated mice (Figure 3C). The increase in CD45 transcript levels observed on day 1 post-siRNA treatment, however, was not sustained through days 3 and 5 in these mice (Figure 3C).

Immune factor production in the genital tract following siRNA instillation

We quantified levels of both human and murine tumor necrosis factor alpha (TNF-α) and interferon alpha (IFN-α) to determine whether inflammatory cytokines were induced in the genital tract following exposure to siRNA (Figure 4). These inflammatory cytokines have been reported to mediate anti-viral effects and could contribute to the inhibition of HIV-1 transmission in mice that received siRNA intra-vaginally. By day 1, human TNF-α transcript levels were increased in the genital tissues from mice that received the combination of siRNAs to CCR5 and CD4 compared to control mice that received glucose. This modest increase, however, was not sustained on subsequent days of analysis (Figure 4A). In mice that received the irrelevant siRNA, levels of human TNF-α transcripts in the genital tissues were not significantly different compared to those of control mice at any time of analysis (Figure 4A). Interestingly, levels of murine TNF-α transcripts increased significantly in both the receptor-specific siRNA and the

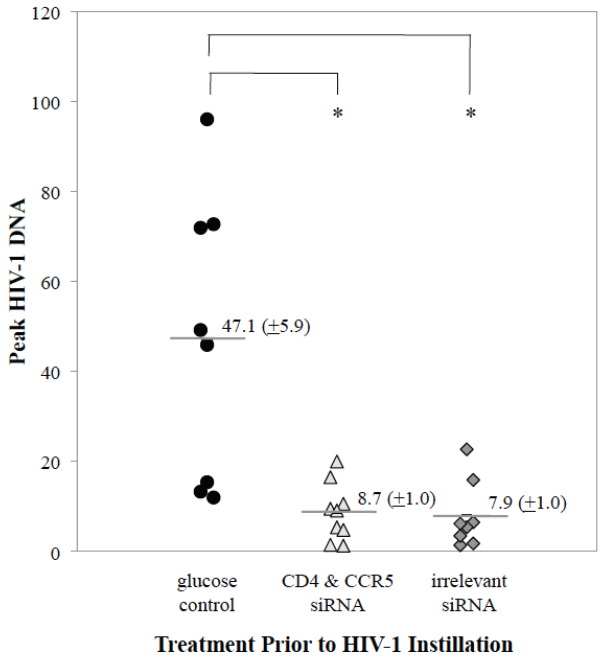

Figure 2

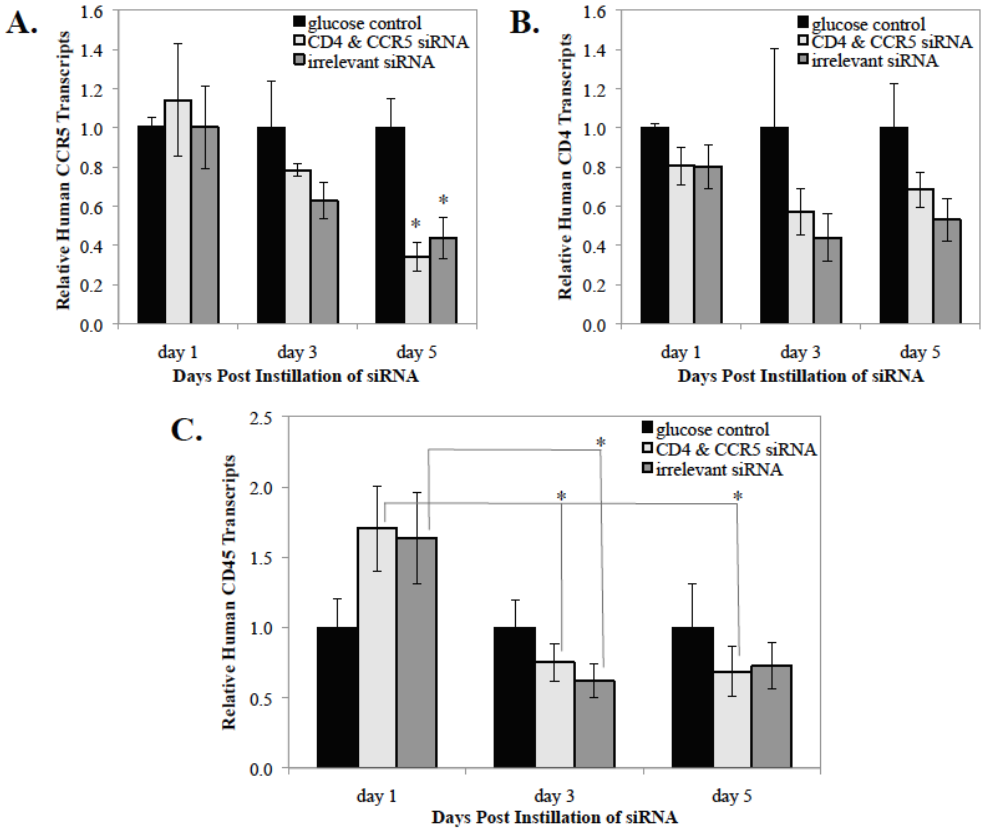

Figure 3

irrelevant siRNA-treated groups, but not until day 5 post-instillation (Figure 4B). Levels of murine IFN-α also increased on day 5 post-siRNA instillation in mice that received the combination of receptor-specific siRNAs (Figure 4B). We were unable to detect expression of human IFN-α, IFN-β, or IFN-γ in genital tissues at any of the time points analyzed, regardless of the treatment.

Anti-viral gene expression in the female genital tract post-siRNA instillation

We measured transcript levels of several anti-viral molecules known to regulate HIV-1 production. These included myxovirus resistance protein 1 (MxA), 2,5-oligoadenylate synthetase (OAS), and protein kinase-A activated (PKR). Human MxA is an interferon-induced dynamin-like GTPase that acts as a cell-autonomous host restriction factor against many viral pathogens [26]. OAS is an enzyme produced by innate immune cells to degrade viral and cellular RNAs and thereby block viral infection [27]. PKR is an interferon stimulated gene that modulates HIV-1 translation via the phosphorylation of the eukaryotic translation initiation factor 2; the phosphorylated factor blocks translation initiation and consequently viral replication [28]. We did not detect any significant increases in expression of these anti-viral genes at any of the time points assayed.

DISCUSSION

HIV-1 is most frequently transmitted following sexual contact with an infected individual. In women, HIV-1 infected cells as well as free viral particles present in the seminal fluid of an infected male partner are the primary sources of infection [29]. Both free virions and cell-associated HIV-1 are deposited in the mucosal tissues of the female genital tract and are transmitted through the mucosal epithelium where they encounter HIV-1 susceptible target cells in the submucosa. These target cells are largely CD4+ macrophages, T lymphocytes, and dendritic cells. Binding of virus to the CD4 and CCR5 receptors on these target cells initiates the infectivity event that ultimately leads to viral transmission to the periphery.

Because CD4 and CCR5 receptors on target cells in the female genital tract are critically important for viral infection, we reasoned that if expression of these receptors were suppressed, HIV-1 infection and viral transmission to the periphery could be inhibited. We previously demonstrated this concept *in vitro* using both peripheral blood mononuclear cells as well as tissue explants derived from human hysterectomy tissues [24]. In this earlier study, nanoparticles encapsulating siRNA specific for CD4 and CCR5 were effective in suppressing viral infection and replication in both peripheral blood cells as well as tissue

Legend to Figure 2. HIV-1 DNA levels in the peripheral blood of mice following siRNA instillation and HIV-1 challenge. Mice received a solution of 5% glucose (glucose control), or siRNAs specific for the CD4 and CCR5 sequence, or an irrelevant sequence intravaginally 3 days prior to challenge with HIV-1. Blood was obtained via tail snip weekly, starting from week 2 and continuing through to week 7. Total genomic DNA was isolated from dried blood spots and HIV-1 DNA levels were measured by PCR and normalized to input DNA with a PCR that detects both human and murine b-actin DNA. Peak HIV-1 DNA levels for each animal, relative to b-actin is plotted in this figure, and the mean (±SEM) for all animals in each group is noted. HIV values are expressed relative to the mean HIV levels detected in the glucose treated mice at week 2, the lowest detected levels. Asterisks denote statistical significance (at $p \leq 0.05$) when comparing the siRNA-treated mice to the glucose control group.

Legend to Figure 3. Expression of CCR5, CD4 and CD45 in genital tract tissues post-siRNA instillation. Mice received intra-vaginal instillation of a 5% glucose solution (glucose control), or siRNAs to CCR5 + CD4, or an irrelevant siRNA sequence. On days 1, 3 and 5 post-application, 3 to 4 mice in each group were euthanized and genital tissues were removed, RNA was isolated, and transcript levels for human CCR5 (panel A), CD4 (panel B), or CD45 (panel C) was measured and normalized to GAPDH with primers that recognize both human and murine GAPDH transcripts. A significant decrease in CCR5 expression was observed on day 5 in tissues from mice that received the receptor-specific and the irrelevant siRNA (panel A). The changes in CD4 transcript levels were not significant, although there was a trend of decreased expression over time (panel B). CD45 expression was elevated on day 1 post-siRNA instillation compared to the glucose control group, but this increase was neither significant nor sustained on subsequent days (days 3 and 5, panel C). Asterisks denote significance at $p \leq 0.05$.

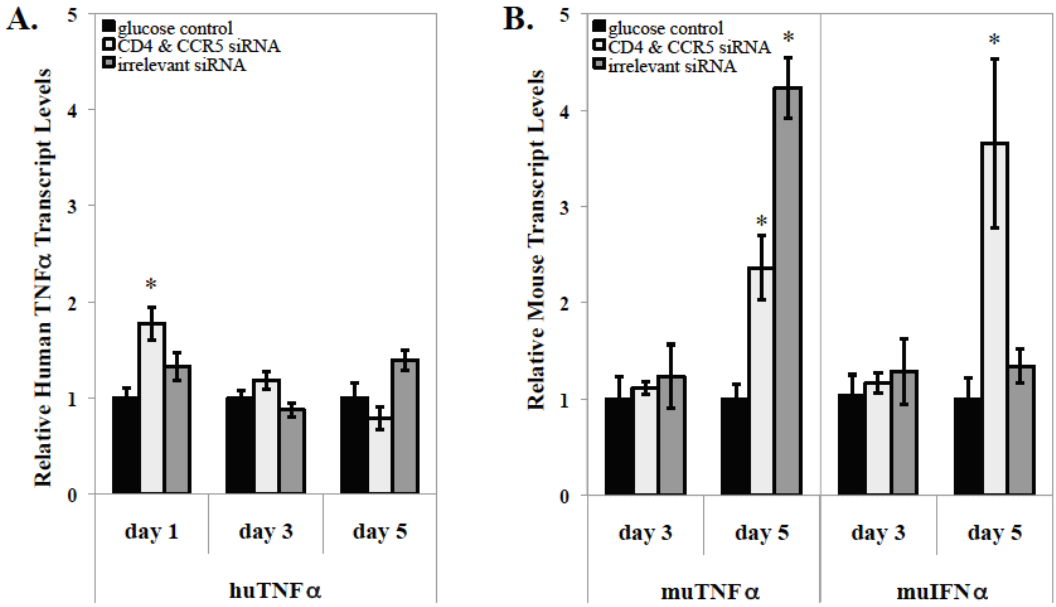

Figure 4. Inflammatory gene expression in genital tissues of siRNA-treated mice. Genital tissues of mice were excised on days 1, 3 and 5 following either glucose or siRNA instillation and total RNA was isolated. Primers to human TNF-α (panel A), or murine TNF-α and IFN-α (panel B) were used to quantify transcript levels that were normalized to GAPDH using primers that recognize both human and murine GAPDH transcripts. We observed significant increases in human TNF-α on day 1 post-instillation of receptor-specific siRNAs (panel A), murine TNF-α on day 5 from mice that received both receptor-specific and irrelevant siRNA (panel B) and murine IFN-α on day 5 from mice that received receptor-specific siRNA (panel B). Asterisks denote significance at $p \leq 0.05$.

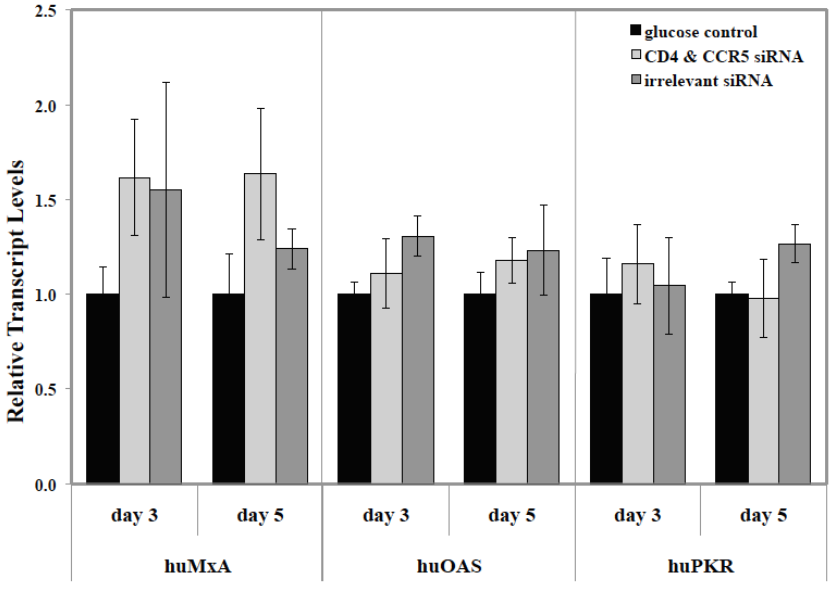

Figure 5. Anti-viral gene expression in genital tissues of siRNA-treated mice. Genital tissues of mice were excised on days 3 and 5 following either glucose or siRNA instillation and total RNA was isolated. Primers to human MxA, OAS and PKR were used to quantify transcript levels that were normalized to GAPDH using primers that recognize both human and murine GAPDH. We did not observe any significant increase in expression of mRNA for these anti-viral molecules in any of the conditions analyzed.

explants from the human endocervix and ectocervix [24]. In these studies, treatment of cells or tissue explants with irrelevant siRNA did not result in the inhibition of HIV-1 replication when compared to media only treatments.

In the current study, we sought to confirm these findings using a humanized mouse model and further explored additional relevant variables an *in vivo* setting provides, such as cellular trafficking and cytokine signaling resulting from siRNA delivery. The humanized mouse provides a relevant pre-clinical model system in which these parameters can be evaluated with respect to mucosal HIV-1 infection and transmission. Female humanized mice received a single intravaginal application of the combination of siRNAs specific for CD4 and CCR5 mRNA. Control mice received either an irrelevant siRNA or a buffer solution. Three days later, the mice were challenged with a single intra-vaginal application of cell-free HIV-1. On subsequent weeks post-infection we used PCR to measure HIV-1 DNA levels in peripheral blood rather than secreted p24 protein to quantify HIV-infected cells. Quantifying HIV-1 DNA is a surrogate marker for the number of HIV-infected cells in the periphery, thus allowing us to specifically measure HIV-1 transmission from the reproductive tract. Our findings show that the instillation of siRNA to the female genital tract, regardless of specificity of the siRNA, was effective in reducing the magnitude of peripheral blood HIV-1 DNA by approximately 6-fold throughout the study period.

As both the irrelevant siRNA and the receptor-specific siRNAs equally reduced the levels of HIV-1 DNA in the peripheral blood, we hypothesize that exposure of the innate immune cells in the female genital tract to siRNA molecules likely induced anti-viral immune responses independent of gene silencing. The primary function of siRNA is to induce RNA interference, a mechanism by which a single-stranded RNA molecule silences expression of a specific messenger RNA. In this process, siRNA enters a cell and becomes incorporated into a multi-subunit protein complex called the RNA interference-induced silencing complex (RISC). RISC then interacts with an appropriate target mRNA that expresses the complementary sequence to the siRNA, upon which the siRNA unwinds the mRNA and then directs the degradation of the complementary strand of mRNA using a combination of endo- and exo-nucleases. In this manner, the mRNA that codes for a particular protein is unable to be translated and its protein product is not expressed. In our study, the intravaginal application of siRNAs targeting both CD4 and CCR5 was expected to reduce cellular expression of these receptors and therefore inhibit the binding and infection of these cells by HIV-1. Although we did observe slight decreases in transcript levels for both CCR5 and CD4 in genital tract tissues on days 3 and 5 post-siRNA instillation in mice that received the receptor-specific siRNAs compared to the glucose only controls, these reductions were also observed in animals that received the irrelevant siRNA; this was an unexpected effect. This data suggests that non-specific effects of siRNA exposure were likely contributing to decreases in CD4 and CCR5 transcript levels in the genital tract. A subset of innate immune receptors including TLR 3, 7, and 8, PKR and OAS among several others, binds foreign RNA triggering downstream signaling cascade events and subsequent cellular migration [30]. These initial immune events could either hinder or augment the potential therapeutic effects of siRNA treatment strategies. Thus, it is important to consider these immune responses secondary to siRNA delivery in the analysis of results [31]. Early successes in the field of anti-viral siRNA therapies are now understood to have substantially benefitted from these non-specific innate immune responses [32-34]. As seen in numerous studies and reviewed in-depth in several publications [35-37], the initial immune response to nucleic acids such as siRNA, regardless of their intended specificity, may be of equal interest and have as much impact as the intended knockdown target. The finding that irrelevant siRNAs were equally effective in reducing CD4 and CCR5 transcripts in genital tract tissues compared to the receptor-specific siRNA could likely be the result of siRNA-induced leukocyte trafficking out of the genital tract upon exposure to siRNA.

To assess whether exposure of the genital tract to siRNA led to altered numbers of leukocytes within this site, we compared human CD45 transcript levels among the three groups of mice. CD45 is a pan-leukocyte receptor expressed by all

lineages of leukocytes [38]. Although we observed an increase in CD45-expression in genital tissues 24 hours after instillation of either the specific or irrelevant siRNAs, this trend was reversed on days 3 and 5 post-siRNA treatment, as CD45 transcript levels were lower in both receptor-specific as well as the irrelevant siRNA-treated groups compared to the control mice. These findings could be explained by an initial influx of CD45+ leukocytes following siRNA application, followed by an efflux of these cells from the genital tract upon cessation of initial innate signaling. We have shown in two recent publications that activation of Toll like receptor-7 and 8 (TLR7, TLR8) induces the production of inflammatory chemokines and cytokines [39, 40]. TLR7 and TLR8 bind to single-stranded RNA molecules and represent one of several classes of innate immune receptors that play an important role in protecting the host from pathogen invasion. As siRNAs are single-stranded RNA molecules, it is likely that they are potent inducers of TLR7 and/or TLR8 activation in genital tissues. This would lead to the induction of expression of inflammatory mediators, chemokines and cytokines, including type 1 interferons that induce the trafficking of immunoreactive cells. Thus, it is possible that innate immune responses to the siRNA could influence both the influx and efflux of immune cells in genital tissues. The finding that CD45 expression in the genital tract is lower in siRNA-treated animals compared to the control animals at the time of HIV-1 exposure (day 3), suggests that there could have been fewer HIV-susceptible target cells present in the genital tract of siRNA-treated cells at the time of viral exposure, which likely contributed to the inhibition of HIV infection observed on subsequent time periods.

The activation of TLR by siRNA molecules also has the potential to induce anti-viral molecules that could contribute to the inhibition of HIV infection and/or replication. Several groups, including ours, have shown that activating the TLR signaling pathways in immune cells using agonists and related small molecules potently inhibits HIV-1 infection and replication in activated lymphocytes and macrophages [39, 41-44]. As the epithelial and stromal cells in the humanized mice are of murine origin, we measured induction of both murine and human type I interferon (IFN-α) and TNF-α. We were unable to detect expression of human IFN-α from any of the animals, perhaps because of the lower expression of these transcripts in human immune cells present in genital tract tissues of these mice. This may also be the case for expression of human TNF-α that was not significantly increased except on day 1 in the receptor-specific siRNA treated group. Overall, the expression of transcripts for human inflammatory molecules was much lower than the expression of the corresponding transcripts of murine origin. Expression of murine IFN-α was significantly increased by day 5 in those animals that received the receptor specific siRNAs, and murine TNF-α was increased on day 5 in all siRNA-treated animals. Expression of MxA, OAS and PKR were not significantly increased following siRNA application; a finding that was not unexpected given the low levels of IFN-α which serves to induce these anti-viral molecules. These findings could also be explained by several other factors, including lower thresholds of induction in our animal model, lower limits of detection in genital tract tissues, or kinetics of induction that may have been different than those time points we assayed.

CONCLUSION

Our studies demonstrate that the intra-vaginal application of siRNA molecules results in a sustained suppression of HIV-1 levels in the peripheral blood of humanized mice, regardless of the specificity of the siRNA. Although it is likely that CD4 and CCR5 receptor expression may play a role in the inhibition of HIV-1 infection and transmission, our data indicate that other mechanisms (possibly innate immune signaling) induced by siRNA contributed to the anti-HIV effect. We did observe temporal alterations in the expression of CD45 indicating that leukocyte trafficking could play a role in the initial infectivity event. We were also surprised that induction of anti-viral molecules including type I interferon did not appear to correlate with the anti-HIV effects, although this could be due to the difficulty inherent in measuring immune-cell specific transcripts by analysis of gene expression from the entire murine genital tract (a “needle in the haystack” phenomenon). More likely to have

impacted HIV transmission was the contribution of murine TNF-α, which has been shown to bind to both murine and human TNF-α receptors [18, 45]. TNF-α not only targets HIV entry in macrophages and inhibits HIV-1 replication in several cell types including monocytes and macrophages [46], but is also capable of down-regulating CD4 and CCR5 expression [19, 45]. TNF-α also induces several HIV suppressive factors in lymphoid cells such as the CCR5 binding ligand RANTES [18, 47]. This finding is further substantiated by our recent observations that CRISPR/Cas RNA transgene delivery to human macrophages via lentiviral vectors (LENTI-LOC™) also induces enhanced levels of TNF-α regardless of the specificity of the targeting guide RNA sequence (manuscript in press).

In sum, further investigation of the mechanisms by which these RNA molecules function to reduce the magnitude of HIV-1 DNA in the peripheral blood of mucosally-infected humanized mice is warranted. Our findings suggest that small biological molecules, including single-stranded RNA molecules that mimic viral genomic material, if properly identified as a contributing factor, could be harnessed to enhance the efficacy of anti-HIV microbicides in mucosal tissues.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article have a financial or scientific conflict of interest with the work reported herein.

ABBREVIATIONS

GAPDH	:	glyceraldehyde 3-phosphate dehydrogenase
HIV-1	:	human immunodeficiency virus
IFN	:	interferon
R5	:	CCR5-tropic HIV-1
siRNA	:	short interfering nucleic acid
TLR	:	Toll like receptor
TNF-α	:	Tumor necrosis factor alpha

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